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㉓ Applicant: **FUJI PHOTO FILM CO., LTD.**  
**210 Nakanuma Minami Ashigara-shi**  
**Kanagawa 250-01(JP)**

㉔ Inventor: **Arai, Fuminori**  
**Fuji Photo Film Co., Ltd. 11-46, Senzui**  
**3-chome**  
**Akasa-shi Saitama(JP)**

㉕ Representative: **Kraus, Walter, Dr. et al**  
**Patentanwälte Kraus, Weisert & Partner**  
**Thomas-Wimmer-Ring 15**  
**D-8000 München 22(DE)**

㉖ **Dry-type analytical element for cholesterol.**

㉗ A dry-type analytical element for the determination of cholesterol comprising at least one liquid-permeable layer and having a porous spreading layer, and containing an enzyme having the hydrolytic activity against cholesterol ester, a bile acid compound and cholesterol dehydrogenase in the liquid-permeable layer.

This analytical element is stable and can be stored for a long time.

**EP 0 244 825 A1**

DRY-TYPE ANALYTICAL ELEMENT FOR CHOLESTEROLBACKGROUND OF THE INVENTIONField of the Invention

5 This invention relates to a dry-type analytical element for the determination of cholesterol particularly total cholesterol including the cholesterol in a bound state in a biological body fluid such as blood or other liquid samples.

Description of Prior Art

The quantitative analysis of cholesterol using cholesterol oxidase is known (Japanese Patent KOKOKU No. 54-8318). In this method, cholesterol is oxidized in the presence of cholesterol oxidase to produce  
15 hydrogen peroxide and cholestenone, and one of the products is determined by a known method.

On the other hand, it is said that cholesterol exists in blood as protein-bound cholesterol ester and free cholesterol ester in addition to free cholesterol. Thus, in order to determine total cholesterol concentration, the cholesterol in the above bound states should be converted to free cholesterol.

The combination of cholesterol esterase with cholesterol oxidase for the purpose of total cholesterol determination is disclosed in USP 3,925,164. In this method, the cholesterol in a bound state is liberated in the presence of cholesterol esterase, and oxidized in the presence of cholesterol oxidase.

In order to determine  $H_2O_2$ , Trinder reagent is useful, but the presence of bilirubin gives an error to the analytical result. As the means to remove the interference of bilirubin, the addition of a ferrocyanate is disclosed in USP 4,291,121. However, when a ferrocyanate is added to a dry-type analytical element  
25 containing cholesterol esterase, cholesterol oxidase, peroxidase and Trinder reagent (or a similar reagent system to detect hydrogen peroxide), the stability of the analytical element during storage is remarkably lowered.

It is also known that, in the case of using cholesterol esterase, another means to decompose the bond between protein and cholesterol ester is necessary. As this means, protease is employed together with  
30 lipase in USP 3,869,349, and a surfactant such as polyethylene glycol alkyl ether is employed together with cholesterol esterase in USP 3,925,164. Furthermore, it is disclosed in USP 4,275,151 and USP 4,274,152 that polyethylene glycol alkylphenyl ethers (alkylphenoxy polyethoxyethanol) having less than 20 ethylene glycol units are effective.

However, when the polyethylene glycol alkyl phenyl ether having less than 20 ethylene glycol units was  
35 incorporated into a porous spreading layer of a dry-type analytical element, spreading area of a liquid sample in the spreading layer became too broad. As a result, some problems such as lowering of sensitivity in colorimetry, lowering of quantitative spreading (proportionality between supplied amount of liquid and spreading area) and insufficient wettability of coating solution to the spreading layer happened. In addition, when this surfactant was incorporated in the layer existing between the spreading layer and a support and  
40 being composed of a hydrophilic polymer binder, such as a water absorption layer, a reagent layer, a light-blocking layer or an adhesive layer, wetting between this layer and another layer was liable to be insufficient. This brought unevenness in coating.

SUMMARY OF THE INVENTION

An object of the invention is to provide a dry-type analytical element for the determination of cholesterol, having a good sensitivity comparable to the conventional dry-type analytical element containing cholesterol esterase, cholesterol oxidase, peroxidase and Trinder reagent, and having a long shelf life.

50 Another object of the invention is to provide a dry-type analytical element for the determination of cholesterol having a suitable spreading area in a definite time and having a high sensitivity to total cholesterol in colorimetry.

Still another object of the invention is to provide a dry-type analytical element for the determination of cholesterol having a good quantitative spreading and capable of uniform coating of a coating solution in its preparation.

As the dry-type analytical element for the determination of cholesterol achieving such objects, the present invention provides a dry-type analytical element for the determination of cholesterol comprising at least one liquid-permeable layer and having a porous spreading layer, and containing an enzyme having the hydrolytic activity against cholesterol ester, a bile acid compound and cholesterol dehydrogenase in the liquid-permeable layer.

The present invention also provides a dry-type analytical element for determination of cholesterol comprising at least one liquid-permeable layer and having a porous spreading layer, and containing an enzyme having the hydrolytic activity against cholesterol ester in the spreading layer and an alkylphenoxypolyglycidol in the liquid-permeable layer.

#### DETAILED DESCRIPTION OF THE INVENTION

The enzyme having the hydrolytic activity against cholesterol ester (cholesterol ester hydrolase) includes cholesterol esterase and lipase. Cholesterol esterase may be obtained from an animal organ or from a microorganism such as Candida rugosa, Actinomycetes, Streptomyces, Penicillium, etc. These microorganisms are disclosed in USP 3,925,164. Lipase may be of plant origin such as wheat germ animal origin such as pancreas or microbial origin such as Candida rugosa. However, the lipase obtained from Chromobacterium viscosum is preferable. Lipase may be combined with cholesterol esterase to use.

In order to raise the reaction rate described dehydrogenase, an oxidized form coenzyme, a dye precursor and an electron transporting carrier substance. In this coloring utilizing this reaction, a dye is produced from a dye includes taurocholic acid, glycocholic acid, taurodeoxycholic acid and glycodeoxycholic acid. The bile acid and its derivative may be a salt such as sodium salt, potassium salt or lithium salt. Among the above compounds, deoxycholic acid and its salt is the most preferable. Hereafter, the bile acid, its derivative and these salts are referred to as bile acid compounds. Effect of the bile acid compound remarkably appears in the case of the coloring system of cholesterol dehydrogenase described below.

Cholesterol may be detected by the following coloring systems.

In the first coloring system, cholesterol dehydrogenase is employed. The reagent composition for this coloring system is composed of cholesterol dehydrogenase, an oxidized form coenzyme, a color precursor and an electron transporting material. In this coloring system, cholesterol is dehydrogenated in the presence of cholesterol dehydrogenase and an oxidized form coenzyme to produce cholestenone, and at that time, the oxidized form coenzyme is converted to its reduced form. By utilizing this reaction, a color is produced from a color precursor.

Preferable cholesterol dehydrogenases are NAD- or NADP-dependent type disclosed in Japanese Patent KOKAI Nos. 53-56090, 58-89200 and 61-108400.

As the oxidized form coenzyme, oxidized form nicotinamide adenine dinucleotide (NAD) and oxidized form nicotinamide adenine dinucleotide phosphate (NADP) are usable.

The color precursor includes various tetrazolium salts such as 3,3'-(3,3'-dimethoxy-4,4'-biphenylene)bis[2-(p-nitrophenyl)-5-phenyltetrazolium chloride] (NBT), 3,3'-(3,3'-dimethoxy-4,4'-biphenylene)bis[2,5-diphenyltetrazolium chloride] (BT), 3-(4',5'-dimethyl-2-triazolyl)-2,4-diphenyltetrazolium bromide (MTT), 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT), 2,2',5,5'-tetra(p-nitrophenyl)-3,3'-(3,3'-dimethoxy-4,4'-biphenylene)ditetrazolium chloride (TNBT), 2,3,5-triphenyltetrazolium chloride (TT) and 3,3'-(4,4'-biphenylene)-bis[2,5-diphenyltetrazolium chloride] (NT). Among these, NBT (common name: Nitrotetrazolium Blue) is preferable.

The electron carrier is diaphorase (E.C. 1, 6, 4, 3), N-methylphenazine methosulfate compound such as N-methylphenazine methoxysulfate or 1-methoxy-N-methylphenazine methosulfate, Meldra Blue, Methylene Blue or the like. Among them, diaphorase and N-methylphenazine methosulfate are preferable.

In the case of using this coloring system, it is preferable that the dye precursor is separated from the carrier, and they are incorporated in different layers from each other. Other components may be incorporated in either of the above layers or other suitable layers. By the above separation, gradual degradation of the reagent composition and fogging during storage decrease. As a result, sensitivity and accuracy are raised.

The following two coloring systems for cholesterol assay using cholesterol oxidase may also be applied to the analytical element of the invention.

The reagent composition for one of the coloring systems is composed of cholesterol oxidase, peroxidase, a chromogen and a coupler. In this coloring system, cholesterol is oxidized in the presence of cholesterol oxidase to produce cholest-4-en-3-one and hydrogen peroxide. A chromogen is oxidized by the hydrogen peroxide to couple with coupler by the hydrogen peroxide in the presence of peroxidase to produce a quinoneimine dye.

The reagent composition for the other coloring system is composed of cholesterol oxidase, peroxidase and a leuco dye or a self-oxidative dye-forming chromogen. In this coloring system, a leuco dye or a self-oxidative dye-forming chromogen is oxidized by hydrogen peroxide in the presence of peroxidase to produce a color compound.

The chromogen includes 4-aminoantipyrine (4-aminophenazone, i.e. 1-phenyl-2,3-dimethyl-4-amino-3-pyrazoline-5-one, described in Ann. Clin. Biochem., 6, 24-27 (1969)), tri-substituted-4-amino-3-pyrazoline-5-one such as 1-(2,4,6-trichlorophenyl)-2,3-dimethyl-4-amino-3-pyrazoline-5-one and 1-(3,5-dichlorophenyl)-2,3-dimethyl-4-amino-3-pyrazoline-5-one disclosed in EP 0103901A and 4-aminoantipyrine analogs such as 1-phenyl-2,3-dimethyl-4-dimethylamino-3-pyrazoline-5-one disclosed in USP 3,886,045. Among these, 4-aminoantipyrine, 1-(2,4,6-trichlorophenyl)-2,3-dimethyl-4-amino-3-pyrazoline-5-one and 1-(3,5-dichlorophenyl)-2,3-dimethyl-4-amino-3-pyrazoline-5-one are preferable.

The coupler includes phenol, phenolsulfonic acid (including alkali metal salts and alkaline earth salts) such as 2-hydroxy-1-benzenesulfonic acid, 4-hydroxy-1-benzenesulfonic acid, 3,5-dichloro-2-hydroxy-1-benzenesulfonic acid and 2-hydroxy-3-methoxy-1-benzenesulfonic acid, 1-naphthol, 2-naphthol, dihydroxynaphthalenes such as 1,7-dihydroxynaphthalene, naphtholsulfonic acids (including alkali metal salts and alkaline earth salts) such as 1-hydroxy-2-naphthalenesulfonic acid and 1-hydroxy-4-naphthalenesulfonic acid, other phenol derivatives and other naphthol derivatives disclosed in Ann. Clin. Biochem., 6, 24-27 (1969), USP-3,886,045, USP-4,012,325, Japanese Patent KOKOKU No. 58-45599 and Japanese Patent KOKAI Nos. 55-164356, 56-124398 and 56-155852. Among these compounds, 1,7-dihydroxynaphthalene, 1-hydroxy-2-naphthalene sulfonic acid (including Na salt, K salt and Li salt) and 3,5-dichloro-2-hydroxy-1-benzenesulfonic acid (including Na salt, K salt and Li salt) are preferable.

The leuco pigment includes triarylimidazole leuco pigments disclosed in Japanese Patent KOKOKU 57-5519 such as 2-(4-hydroxy-3,5-dimethoxyphenyl)-4,5-bis[4-(dimethylamino)phenyl]imidazole, diarylimidazole leuco pigments disclosed in Japanese Patent KOKAI No. 59-193352 such as 2-(3,5-dimethoxy-4-hydroxyphenyl)-4-[4-(dimethylamino)phenyl]-5-phenethylimidazole, diarylindolylimidazole leuco pigments disclosed in Japanese Patent KOKAI No. 61-4960 such as 2-(2-phenyl-3-indolyl)-4,5-di[4-(dimethylamino)phenyl]imidazole, triarylmonoacylimidazole leuco pigments disclosed in Japanese Patent KOKAI No. 61-229868 such as 2-(4-hydroxy-3,5-dimethoxyphenyl)-3-acetyl-4,5-bis[4-(dimethylamino)phenyl]imidazole, and triarylmonoalkylimidazole leuco pigments such as 2-(4-hydroxy-3,5-dimethoxyphenyl)-3-methyl-4,5-bis[4-(diethylamino)phenyl]imidazole.

Among the above three coloring systems, the first coloring system containing cholesterol dehydrogenase is the most preferable.

Other reagents may be added according to kind of the sample etc. Such reagents include a nonionic surfactant, a sensitivity moderator and a buffer.

The nonionic surfactant includes a polyhydric alcohol ester ethylene oxide adduct (condensate), a polyethylene glycol monoester, a polyethylene glycol diester, a higher alcohol ethylene oxide adduct (condensate) and an alkylphenol ethylene oxide adduct (condensate).

Examples of the nonionic surfactant are disclosed in USP 3,925,164, USP 3,983,005, USP 4,275,152 and USP 4,275,151, and include,

POE (10) sorbitan monooleate  
 PEG (400) monostearate  
 Lauryl alcohol EO 10 moles condensate  
 POE (10) octylphenyl ether  
 POE (15) octylphenyl ether  
 POE (12) nonylphenyl ether  
 Hydroxypolyethoxydodecane  
 (Note) POE: Polyethylene oxide  
 PEG: Polyethylene glycol  
 EO: Ethylene oxide

The number in parentheses represents condensation number of ethylene oxide units

As the nonionic surfactant, an alkylphenoxypolyglycidol is preferable. Preferable carbon number of the alkyl group is 4 to 20. The above benzene ring may be substituted by two or more alkyl groups, that is, a dialkylphenoxypolyglycidol is also included. The benzene ring may contain other groups such as an alkoxy group or a halogen atom. The number of glycidol units is usually 7 to 20.

5 The content per square meters of each component in the analytical element of the invention is as follows:

The content of cholesterol esterase is about 500 to about 50,000 IU preferably about 1,000 to 30,000 IU.

When lipase is used, the content of lipase is about 5,000 U to about 300,000 U preferably 10,000 to about 200,000 U.

10 The content of the bile acid compound is about 200 mg to about 10 g preferably about 500 mg to about 5 g.

When the first coloring system is employed, the content of cholesterol dehydrogenase is about 200 to about 30,000 U preferably about 300 to 20,000 U. The content of the oxidized form coenzyme is about 10 mg to about 50 g preferably about 30 mg to about 20 g. The content of the color precursor is about 5 mg to about 10 g preferably about 25 mg to about 5 g, and the content of the electron transporting material is about 1 mg to about 1,000 mg preferably about 5 mg to about 500 mg. When diaphorase is employed as the electron transporting material, the content of diaphorase is about 500 to about 20,000 U preferably about 700 to about 10,000 U.

When the other coloring system is employed, the content of cholesterol oxidase is about 1,000 to about 30,000 IU preferably about 1,500 to about 20,000 IU. The content of peroxidase is about 1,000 to about 100,000 IU preferably about 2,000 to about 60,000 IU, and contents of the chromogen and the leuco pigment are about 0.5 to about 10 mmol. preferably about 1 to about 5 mmol. The content of the coupler is about 2.5 to about 50 mmol. preferably about 5 to about 25 mmol. The content of the alkylphenoxypolyglycidol is about 200 mg to about 10 g preferably about 500 mg to about 5 g.

25 The dry-type analytical element of the invention comprises at least one liquid-permeable layer, and has a porous spreading layer. The liquid-permeable layer may be porous spreading layer, reagent layer containing dye-forming layer, light-blocking layer, water absorption layer, or the like, however, the porous spreading layer is essential for the analytical element of the invention.

The analytical element may be multilayer, and it may contain various known layers, such as undercoat layer, water absorption layer, adhesive layer, reagent layer, light-blocking layer and filtering layer, in addition to the spreading layer and the light-transmissive water-impermeable support. Examples of such a multilayer analytical element are disclosed in USP 3,992,158 and Japanese Patent KOKAI No. 55-164356.

The multilayer analytical element containing a support of the invention includes the following embodiments.

35 (1) A spreading layer and a support. A water absorption layer may be provided therebetween.

(2) A spreading layer, a reagent layer and a support superposed in this order. A water absorption layer may be provided between the reagent layer and the support. The cholesterol ester hydrolase may be incorporated in either of the spreading layer or the reagent layer, however the spreading layer is preferable.

40 (3) A spreading layer, a first reagent layer, a second reagent layer and a support. A water absorption layer may be provided between the second reagent layer and the support. The cholesterol ester hydrolase may be incorporated in any layer of the spreading layer, the first reagent layer or the second reagent layer, however the first reagent layer or the spreading layer is preferable.

(4) A spreading layer, a light-blocking layer, a reagent layer and a support superposed in this order. A water absorption layer may be provided between the reagent layer and the support. The cholesterol ester hydrolase may be incorporated in either of the spreading layer or the reagent layer, however the spreading layer is preferable.

50 (5) A spreading layer, a first reagent layer, a light-blocking layer, a second reagent layer and a support superposed in this order. A water absorption layer may be provided between the second reagent layer and the support. The cholesterol ester hydrolase may be incorporated in any layer of the spreading layer, the first reagent layer or the second reagent layer, however the spreading layer or the first reagent layer is preferable.

In the above embodiments, an adhesive layer may be provided on the surface of the layer located under the spreading layer for laminating it. The adhesive layer may be provided between other layer. A filtering layer may be provided between the spreading layer or the reagent layer and the water absorption layer, between the spreading layer and the reagent layer, between the first reagent layer and the second reagent layer, or between the light-blocking layer and the reagent layer or the spreading layer.

As the light-transmissive water-impermeable support, a known support employed in an usual multilayer analytical element may be employed. Such a support is a sheet or a laminate having a thickness in the range from about 50  $\mu\text{m}$  to about 1 mm, preferably from about 80  $\mu\text{m}$  to about 0.3 mm, and being clear in the range from near-ultraviolet to near infrared regions. Such a sheet or a laminate may be made of a polyester (for example, polyethylene terephthalate or polycarbonate of bisphenol A), a cellulose ester (for example, cellulose triacetate or cellulose acetate propionate) or polystyrene. A known undercoat layer may be provided on the surface of the support in order to secure the adhesion of the layer provided on the support such as a reagent layer or a water absorption layer to the support. Instead of the undercoat layer, surface of the support may be treated by physical activation such as flow discharge or corona discharge or by chemical activation in order to raise adhesive force.

The spreading layer preferably has liquid metering action. This liquid metering action means that the liquid sample spotted on the surface of this layer uniformly spreads in the transverse direction at the rate of almost equal amount per unit area without uneven distribution of any component. The material constituting matrix of the spreading layer may be filter papers, nonwoven fabrics, woven fabrics such as plain weaves including broad cloth and poplin, knitted fabrics such as tricot, double tricot and milanese, glass fiber filter papers, membrane filters formed out of blushed polymer, three-dimensional lattice structure material or the like. Among them, fibrous materials such as woven fabrics and knitted fabrics are preferable. The spreading layers using the above materials are disclosed in the specifications of Japanese Patent KOKAI Nos. 55-164356, 57-66359 and 60-222769. It is preferable that greases adhered on woven fabrics and knitted fabrics during their manufactures are substantially removed by degreasing such as washing.

The reagent layer contains a part of or whole reagents, and it is non-porous and water-absorptive or microporous and water-permeable.

A hydrophilic polymer binder which is employed for the substantially non-porous and water-absorptive reagent layer functions as a medium for dissolving or dispersing the reagents uniformly. It also functions to absorb the water in a sample and to carry the analyte together with the water. The hydrophilic polymer binder absorbs water to swell, and swelling ratio at water absorption of the polymer binder suitable for the reagent layer is generally about 1.5 to 20 times, preferably about 2.5 to 15 times at 30°C. Examples of the hydrophilic polymer binder usable for the reagent layer are gelatin including alkali-treated gelatin, acid-treated gelatin and deionized gelatin, gelatin derivatives such as phthalated gelatin, agarose, pullulan, pullulan derivatives, polyacrylamide, polyvinyl alcohol and polyvinyl pyrrolidone. Thickness of the reagent layer in dry state is usually about 1  $\mu\text{m}$  to about 100  $\mu\text{m}$ , preferably about 3  $\mu\text{m}$  to about 30  $\mu\text{m}$ .

The microporous and water-permeable reagent layer comprises a microporous structure layer constructed by solid particulates and a hydrophilic polymer binder as the binder thereof and reagent(s) or a reagent composition contained therein. The microporous structure layer referred herein has continuous microspaces structure composed of microporous or non-porous particulates and a hydrophilic polymer binder joining them.

Examples of the microporous or non-porous particulates include cellulose particulates such as microcrystalline cellulose and cellulose fine powder, particulates of silicon dioxide compound such as silica and diatomaceous earth, silicate particulates such as zeolite, polymer particulates, glass particulates, and various ceramic particulates. The hydrophilic polymer binder may be selected from the illustrated above or aqueous latex of copolymer containing more than 2% of hydrophilic repeating unit disclosed in EP 0,115,873 A. Thickness of the microporous reagent layer in dry state is about 7  $\mu\text{m}$  to about 50  $\mu\text{m}$ , preferably about 10  $\mu\text{m}$  to about 30  $\mu\text{m}$ .

The reagent layer may be incorporated with a pH buffer composition, a macromolecular pH buffer, a base polymer, an acid polymer, a macromolecular mordant, etc., to be known. The pH buffer composition may be a carbonate buffer, a borate buffer, a phosphate buffer, Good's buffer or the like.

The light-blocking layer is water-transmissive or water-permeable, and light-shielding particulates or the particulates having both functions of light-shielding and light-reflecting are disposed in and held by a small amount of a hydrophilic polymer binder capable of forming a film. The light-blocking layer shields color of an aqueous liquid sample, particularly red color of hemoglobin in a whole blood sample, spotted on the spreading layer during measuring the color development from the side of the light-transmissive support. This layer may also function as a light-reflecting layer or a background layer.

Examples of the particulates having both functions of light-shielding and light-reflecting are titanium dioxide particulates such as rutile, anatase and brookite microcrystalline particulates having a particle size of about 0.1  $\mu\text{m}$  to about 1.2  $\mu\text{m}$ , barium sulfate particulates and aluminum particulates and microflakes. Examples of the light-shielding particulates are carbon black, gas black and carbon microbeads. Among them, titanium dioxide particulates and barium sulfate particulates are preferable. The hydrophilic polymer

binder having film-forming property may be selected from those illustrated in the foregoing reagent layer, weakly hydrophilic regenerated cellulose and cellulose acetate. Among them, gelatin, a gelatin derivative and polyacrylamide are preferable. To gelatin and a gelatin derivative, a known curing agent (a cross-linking agent) may be blended.

5 The light-blocking layer is provided by applying an aqueous suspension of light-shielding particulates containing a hydrophilic polymer binder by means of a known method, and then dried. Volume ratio of light-shielding particulates to hydrophilic polymer binder in dry state are light-shielding particulates 10 : hydrophilic polymer binder about 2.5-about 7.5, preferably about 3.0-about 6.5. In the case that the light-shielding particulates are titanium dioxide particulates, the ratio by weight of polymer binder is about 0.6-  
10 about 1.8, preferably about 0.8-about 1.5, per 10 of titanium dioxide. Thickness of the light-blocking layer in dry state is about 3  $\mu\text{m}$  to about 30  $\mu\text{m}$ , preferably about 5  $\mu\text{m}$  to about 20  $\mu\text{m}$ .

The water absorption layer is composed of a hydrophilic polymer binder, and it may contain a cationic, ampholytic or nonionic surfactant and a pH buffer composition. The hydrophilic polymer binders of both layers may be selected from the illustrated previously. Thickness of these layers is dry state are usually  
15 about 1  $\mu\text{m}$  to about 100  $\mu\text{m}$ , preferably about 3  $\mu\text{m}$  to about 30  $\mu\text{m}$ .

The adhesive layer usually comprises the hydrophilic polymer binder to adhere the spreading layer at the wetting state or the swelling state of the polymer. The hydrophilic polymer binder usable for the adhesive layer may be selected from the illustrated in the foregoing water absorption layer, and gelatin, a gelatin derivative and polyacrylamide are preferable. Thickness of the adhesive layer in dry state is about 0.5  
20  $\mu\text{m}$  to about 20  $\mu\text{m}$ , preferably about 1  $\mu\text{m}$  to about 10  $\mu\text{m}$ . A surfactant may be added to the adhesive layer. A nonionic surfactant, particularly having a chain structure of 8-15 hydroxyethylene or hydroxypropylene units is preferable.

The adhesive layer may be provided by applying a solution containing the hydrophilic polymer binder and a surfactant added by request by means of a known method.

25 In the multilayer analytical element of the invention, the enzyme having the hydrolytic activity against cholesterol ester, the bile acid compound, cholesterol dehydrogenase, cholesterol oxidase and alkylphenoxypolyglycidol are preferably incorporated in a upper layer, and spreading layer is the most preferable. However, cholesterol dehydrogenase and cholesterol oxidase may be incorporated the layer just under the spreading layer such as adhesive layer, light-blocking layer or first reagent layer. While, the bile acid  
30 compound is preferably incorporated in spreading layer together with the enzyme having the hydrolytic activity against cholesterol ester and nonionic surfactant such alkylphenoxypolyethoxyethanol. Oxidized form coenzyme, color precursor, electron transporting material, chromogen, leuco pigment and coupler are preferably incorporated in an under layer such as reagent layer. pH buffer may be incorporated in any layer.

35 The integral multilayer analytical element manufactured by the invention is preferably cut into square or circular pieces having a side or diameter of about 15 mm to about 30 mm, and put in a slide frame disclosed in Japanese Patent KOKAI No. 57-63452, USP 4,169,751, USP 4,387,990, PCT application WO 83/00391, etc. to use.

The measurement is carried out as follows. About 5  $\mu\text{l}$  to about 30  $\mu\text{l}$ , preferably about 8  $\mu\text{l}$  to about  
40 15  $\mu\text{l}$  of an aqueous sample is spotted on the spreading layer, and incubated at a definite temperature in the range of about 20°C to about 45°C for a prescribed time, if necessary. Thereafter, detectable variation such as color change or coloring in the multilayer analytical element is measured from the side of the support through reflection photometry, and the subject component in the sample is determined by the principle of colorimetry.

## EXAMPLES

### Example 1

50 A colorless transparent polyethylene terephthalate (PET) film having a thickness of 180  $\mu\text{m}$  on which gelatin undercoating was provided was employed as the support. On the support, the following solution was applied, and dried to form a dye-forming layer having a dry thickness of 15  $\mu\text{m}$ .

Alkali-treated gelatin 12 g

55 Nonylphenylglycidyl ether 0.1 g

NAD 0.8 g  
 Diaphorase 2800 IU  
 NBT\* 0.2 g  
 Water 95 ml

5 \* 3,3'-(3,3'-dimethoxy-4,4'-biphenylene)bis-[2-(p-nitrophenyl)-5-phenyl-2H tetrazolium chloride]

On the dye-forming layer, the following suspension was applied, and dried to form a light-blocking layer having a thickness of 7  $\mu\text{m}$ .

Alkali-treated gelatin 14 g  
 Rutile type titanium dioxide particulates 70 g

10 Water 100 ml

On the light-blocking layer, an aqueous gelatin solution was applied, and dried to form an adhesive layer having a thickness of 2  $\mu\text{m}$ .

The adhesive layer was dampened with 30 g/m<sup>2</sup> of water. A PET tricot fabric cloth having 0.25 mm in thickness was made hydrophilic by glow discharge, and pressed to laminate thereon as a spreading layer,

15 and then dried.

On the spreading layer, the following suspension was applied at the rate of 200 ml/m<sup>2</sup>, and dried to form an integral multilayer analytical element for the determination of cholesterol.

Ethanol 500 ml  
 Polyvinylpyrrolidone K90 18 g  
 20 Polyoxyethylene nonylphenyl ether (n=40) 6 g  
 2-Amino-2-ethyl-1,3-propanediol 11 g  
 Polyoxyethylene octylphenyl ether (n=10) 3 g  
 Sodium deoxycholate 7 g

The following enzyme solution was added to the above ethanol solution to obtain a suspension.

25 Lipase\* 15000 U  
 Cholesterol dehydrogenase 5000 U  
 Water 5 ml

\* Produced by Chromobacterium viscosum

30 The analytical element thus obtained was cut into square pieces of 15 x 15 mm, and each piece was placed in a plastic mount.

Each 10  $\mu\text{l}$  of human sera having various cholesterol concentrations was spotted on the above analytical element, and incubated at 37°C for 6 minutes. Then, reflection optical density was measured by using light of 600 nm from the PET support side. The results are shown in Table 1.

Table 1

	<u>Cholesterol Concentration</u>	<u>Reflection Optical Density</u>
40	62 mg/dl	0.254
	95	0.321
45	158	0.412
	294	0.653
	348	0.721

50

#### Example 2

55 The following solution was applied on a PET film having a thickness of 180  $\mu\text{m}$  on which gelatin undercoating was provided, and dried to form a dye-forming layer having a dry thickness of 15  $\mu\text{m}$ .

Alkali-treated gelatin 12 g  
 Nonylphenylglycidyl ether 0.1 g



Diaphorase 3000 U

NAD 0.35 g

Bis(vinylsulfonylethyl) ether 0.12 g

Water 105 g

- 5 On the dye-forming layer, the following suspension was applied, and dried to form a light-blocking layer having a dry thickness of 7  $\mu\text{m}$ .

Alkali-treated gelatin 7 g

Rutile type titanium dioxide particulates 35 g

Water 50 ml

- 10 On the light-blocking layer, the following solution was applied, and dried to form an adhesive layer containing cholesterol dehydrogenase having a thickness of 4  $\mu\text{m}$ .

Cholesterol dehydrogenase 39000 U

NAD 0.9 g

Alkali-treated gelatin 20 g

- 15 Nonylphenylglycidyl ether 0.2 g

Water 220 g

The adhesive layer was dampened with 30 g/m<sup>2</sup> of water. A PET tricot fabric cloth having 0.25 mm in thickness was made hydrophilic by glow discharge, and pressed to laminate thereon as a spreading layer, and then dried.

- 20 On the spreading layer, the following suspension was applied at the rate of 200 ml/m<sup>2</sup>, and dried to form an integral multilayer analytical element for the determination of cholesterol.

Ethanol 500 ml

Polyvinylpyrrolidone K90 18g

Polyoxyethylene nonylphenyl ether (n = 40) 6 g

- 25 2-Amino-2-methyl-1,3-propanediol 11 g

Polyoxyethylene octylphenyl ether (n = 10) 3 g

Sodium deoxycholate 7g

The following enzyme solution was added to the above ethanol solution to obtain a suspension.

Lipase\* 15000 U

- 30 Water 3 g

\* Produced by Chromobacterium viscosum

The analytical element thus obtained was cut into square pieces of 15 x 15 mm, and each piece was placed in a plastic mount.

- Each 10  $\mu\text{l}$  of the human sera measured in Example 1 was spotted on the above analytical element, and the reflection optical density of each analytical element was measured in the same manner as Example 1. The results are shown in Table 2.

Table 2

	<u>Cholesterol Concentration</u>	<u>Reflection Optical Density</u>
45	62 mg/dl	0.330
	95	0.402
50	158	0.565
	294	0.928
	348	1.08

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Example 3

12,000 U of cholesterol esterase was used instead of 15,000 U of lipase, and an analytical element for the determination of cholesterol was prepared in the same manner as Example 2. In the case of this analytical element, similar results to Example 2 were obtained.

Example 4

In Example 1, 0.3 g of nonylphenoxypolyglycidol ( $n=10$ ) was used instead of 0.1 g of nonylphenylglycidyl ether in the solution for dye-forming layer, and 3 g of nonylphenoxypolyglycidol ( $n=10$ ) was used instead of 3 g of polyoxyethylene octylphenyl ether ( $n=10$ ) in the solution for applying on the spreading layer. Thus, an integral multilayer analytical element for the determination of cholesterol was prepared in the same manner as Example 1.

This analytical element was cut into square pieces of 15 x 15 mm, and each piece was placed in a plastic mount.

Each 10  $\mu$ l of the human sera measured in Example 1 was spotted on the above analytical element, and the reflection optical density of each analytical element was measured in the same manner as Example 1. The results are shown in Table 3.

Table 3

<u>Cholesterol Concentration</u>	<u>Reflection Optical Density</u>
62 mg/dl	0.26
95	0.32
158	0.41
294	0.65
348	0.72

Example 5

In Example 2, 0.1 g of nonylphenylpolyglycidol and 0.35 g of NBT were employed instead of 0.1 g of nonylphenylglycidyl ether and 0.35 g of NAD in the solution for dye-forming layer, and 0.2 g of nonylphenylpolyglycidol ( $n=10$ ) was employed instead of 0.2 g of nonylphenylglycidyl ether in the solution for adhesive layer. Furthermore, 3 g of nonylphenylpolyglycidol ( $n=10$ ) was employed instead of 3 g of polyoxyethylene octylphenyl ether ( $n=10$ ) in the solution for applying on the spreading layer. Thus, an integral multilayer analytical element for the determination of cholesterol was prepared in the same manner as Example 2.

This analytical element was cut into square pieces of 15 x 15 mm, and each piece was placed in a plastic mount. Each 10  $\mu$ l of the human sera measured in Example 1 was spotted on the above analytical element, and the reflection optical density of each analytical element was measured in the same manner as Example 1. The results are shown in Table 4.

Table 4

5	<u>Cholesterol Concentration</u>	<u>Reflection Optical Density</u>
	62 mg/dl	0.330
	95	0.402
10	158	0.565
	294	0.928
15	348	1.08

Example 6

20 12,000 U of cholesterol esterase was used instead of 15,000 U of lipase, and an analytical element for determination of cholesterol was prepared in the same manner as Example 5. In the case of this analytical element, similar results to Example 5 were obtained.

25 **Claims**

1. A dry-type analytical element for the determination of cholesterol comprising at least one liquid-permeable layer and having a porous spreading layer, and containing an enzyme having the hydrolytic activity against cholesterol ester, a bile acid compound and cholesterol dehydrogenase in said liquid-permeable layer.

2. The dry-type analytical element of claim 1 wherein said enzyme having the hydrolytic activity against cholesterol ester is cholesterol esterase which is incorporated in said porous spreading layer.

3. The dry-type analytical element of claim 1 wherein said enzyme having the hydrolytic activity against cholesterol ester is lipase which is incorporated in said porous spreading layer.

35 4. The dry-type analytical element of claim 3 wherein said lipase is produced by Chromobacterium viscosum.

5. The dry-type analytical element of claim 1 wherein said bile acid compound is incorporated in said porous spreading layer.

40 6. The dry-type analytical element of claim 5 wherein said bile acid compound is deoxycholic acid or its salt.

7. The dry-type analytical element of claim 1 wherein said cholesterol dehydrogenase is incorporated in said porous spreading layer.

8. The dry-type analytical element of claim 3 wherein said bile acid component is also incorporated in said porous spreading layer.

45 9. The dry-type analytical element of claim 8 wherein said cholesterol dehydrogenase is further incorporated in said porous spreading layer.

10. The dry-type analytical element of claim 8 or claim 9 wherein said lipase is produced by Chromobacterium viscosum.

50 11. A dry-type analytical element for the determination of cholesterol comprising at least one liquid-permeable layer and having a porous spreading layer, and containing an enzyme having the hydrolytic activity against cholesterol ester and an alkylphenoxypolyglycidol in said liquid permeable layer.

12. The dry-type analytical element of claim 11 which further contains a bile acid compound and cholesterol dehydrogenase in said liquid-permeable layer.

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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X,D	US-A-3 983 005 (GOODHUE et al.) * Column 2, lines 46-54; column 18, line 64 - column 19, line 35 *	1-3,11	G 01 N 33/52 C 12 Q 1/60
X	--- EP-A-0 019 253 (MILES LABORATORIES INC.) * Page 23, lines 6-19; page 24, lines 8-13 *	1,2,4,10	
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A	--- US-A-4 042 329 (HOCHSTRASSER et al.) * Column 3, lines 20-31; column 14, lines 61-68 *	1	
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The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 10-08-1987	Examiner VAN BOHEMEN C.G.
<p><b>CATEGORY OF CITED DOCUMENTS</b></p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons &amp; : member of the same patent family, corresponding document</p>			



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Place of search THE HAGUE		Date of completion of the search 10-08-1987	Examiner VAN BOHEMEN C.G.
<b>CATEGORY OF CITED DOCUMENTS</b>			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			